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Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by hamster, mouse and rat intestine: relevance of species differences

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We recently demonstrated the metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in rat intestinal segments, as well as the inducibility of intestinal NNK metabolism by starvation or acetone treatment. To improve our understanding of intestinal NNK turnover we have additionally investigated NNK metabolism in isolated perfused jejunal segments from NMRI mice and Syrian golden hamsters. [^{14}C]NNK (1 μmol/l) was metabolized extensively by jejunal segments from female NMRI mice (88.5%) and female Syrian hamsters (86.4%), whereas in male NMRI mouse segments a slightly lower metabolism (68.8%) was observed. α-Hydroxylation was the predominant metabolic pathway in mice (58% of total metabolism), whereas in female Syrian hamsters *N*-oxidation accounted for >50% of the metabolites [4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanol 27%, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone 22% of total radioactivity]. Formation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) was low in both species. Total NNK metabolism in male NMRI mice was increased by starvation to 84.4% and by acetone treatment to 90.0% of the absorbed radioactivity. This increase was due to an increase in *N*-oxidation, whereas the amounts of α-hydroxides and NNAL remained unchanged. In female Syrian hamsters acetone treatment had only minimal effects upon the metabolite composition. Acetone-treated NMRI mice and Syrian hamsters were additionally gavaged with the chemopreventive agent phenethylisothiocyanate (PEITC). In mice this treatment slightly decreased keto acid formation (0.6-fold, $P < 0.05$), whereas in hamsters PEITC had no effect. In summary, intestinal metabolism of NNK in rats, mice and hamsters differs in both the extent of total metabolism (hamsters ≥ mice > rats) and the metabolite composition, indicating major species differences.

Introduction

4-Methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK*), a tobacco-specific nitrosamine, is a potent lung carcinogen in rodents (1). It is found in cigarettes and cigarette smoke, but also in other tobacco products, like wet and dry snuff, sometimes in even higher concentrations than in cigarette tobacco (2–4). Whereas smoking is a strong risk factor for lung cancer (5), chewing and snuff taking have been correlated mainly with carcinomas of the oral cavity and the pharynx (6).

NNK can be metabolized by a variety of pathways, including keto reduction, pyridinium *N*-oxidation and α-hydroxylation (see Figure 1). α-Hydroxylation forms intermediates which can react with cellular macromolecules (for a review see 1). DNA and hemoglobin adducts from NNK have been detected in both experimental animals and smokers (1). Reaction with water leads to the formation of 4-hydroxy-4-(3-pyridyl)butanol(diol), 4-oxo-4-(3-pyridyl)-butanol (keto alcohol) and their oxidation products 4-hydroxy-4-(3-pyridyl)butyric acid (hydroxy acid) and 4-oxo-4-(3-pyridyl)butanol (keto acid), which account for 50–74% of the urinary metabolites (7–10). The *N*-oxidation products 4-methylnitrosamino-1-(3-pyridyl-*N*-oxide)-1-butanone (NNK *N*-oxide) and 4-methylnitrosamino-1-(3-pyridyl-*N*-oxide)-1-butane (NNAL *N*-oxide) are also formed *in vivo* in considerable amounts and account for 4.3–34% of the NNK metabolites in the urine of mice and rats (7,9,10). NNK *N*-oxide is much less carcinogenic than NNK (11). Therefore, formation of *N*-oxides is regarded as a detoxification pathway (1). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the product of NNK keto reduction, is a potent pulmonary and pancreatic carcinogen (1,11).

Species differences in NNK turnover rates and metabolite pattern have been demonstrated in lung and liver microsomes from a variety of species. In mouse and rat lung microsomes rates of α-hydroxylation and *N*-oxidation are similar (12–14), whereas in hamster lung α-hydroxylation appears to be favored (15,16). In human lung microsomes NNAL accounts for >95% of all metabolites (17). Similar differences have been detected in liver microsomes (12–14), as well as in antibody inhibition experiments in both lung and liver microsomes (17,18).

Besides hepatic NNK metabolism pulmonary metabolism has been investigated extensively, because the lung is the target organ for NNK-mediated tumorigenesis, as well as the first organ to come into contact with inhaled NNK in cigarette smokers. The intestine has received much less attention, although in snuff takers and tobacco chewers, and partly in smokers, NNK is swallowed and absorbed in the gastrointestinal tract.

Our laboratory has recently reported on the intestinal metabolism of NNK in Sprague–Dawley and Fischer F344 rats (19) and NMRI mice (20). Whereas in rats all three metabolic pathways are present, with NNAL and *N*-oxides as the main metabolites, α-hydroxylation appears to be the predominant activation pathway in mouse intestine (20). Using a different method Pepin *et al.* (21) reported NNAL to be the major metabolite in mouse duodenum and stomach. These species and organ differences prompted us to further investigate species differences in the intestinal metabolism of NNK.

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; keto alcohol, 4-oxo-4-(3-pyridyl)butanol; hydroxy acid, 4-hydroxy-4-(3-pyridyl)butyric acid; keto acid, 4-oxo-4-(3-pyridyl)butyric acid; NNK *N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone; NNAL *N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanol; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; PEITC, phenethylisothiocyanate.

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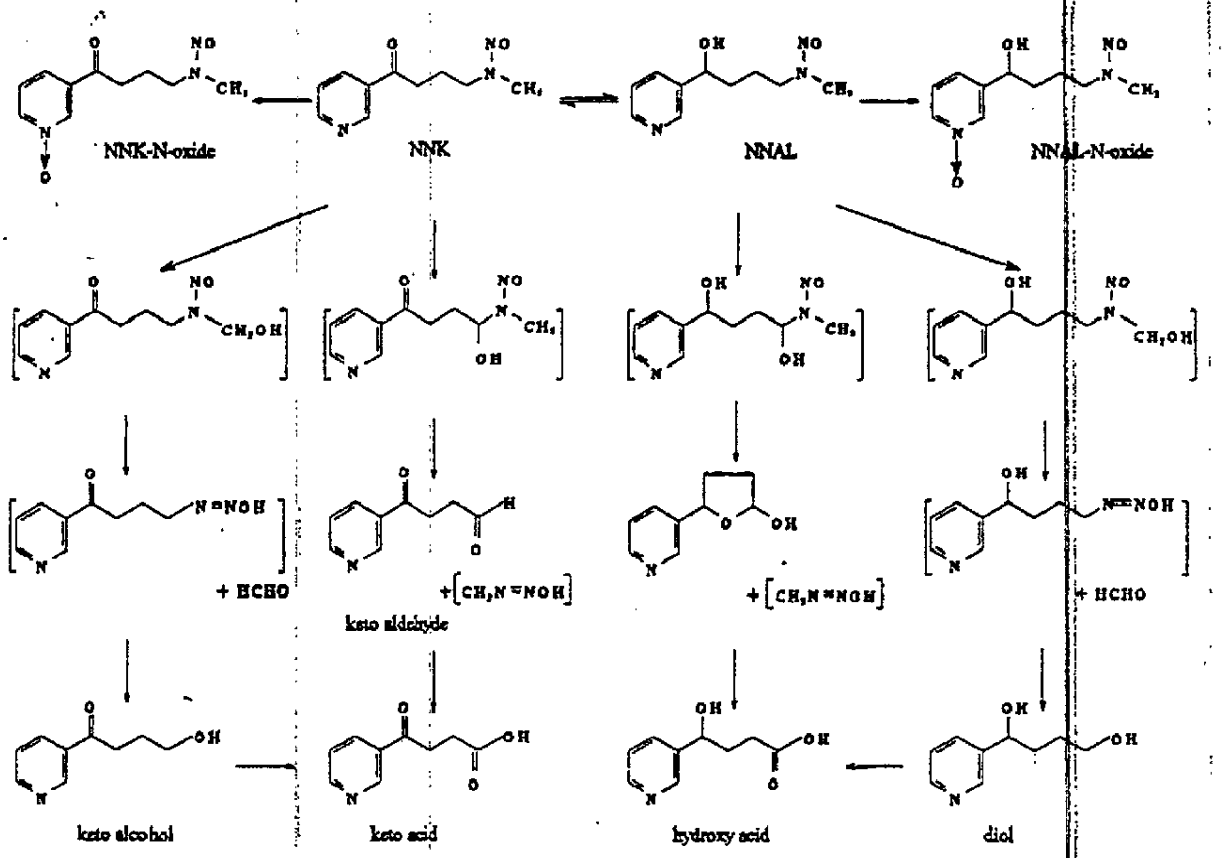

Fig. 1. Metabolism of NNK (from ref.1, modified). Abbreviations refer to those used in the text. Compounds in brackets are unstable intermediates.

Table I. Viability criteria for isolated perfused jejunal segments from NMRI mice and Syrian hamsters

Treatment	n	Water transport (µl/cm/2 h)	Glucose transport (mmol/cm/2 h)	Glucose accumulation	Radioactivity accumulation
Male NMRI mouse					
Control	7	97±80	7.5±0.4	4.8±0.4	1.9±0.1
Starved	9	214±20[b]	15.9±1.0[b]	5.4±0.3[a]	2.2±0.1[b]
Acetone	6	130±10[b]	11.2±1.0[b]	6.8±0.6[b]	2.3±0.7
PEITC	7	142±18[b]	9.5±1.6[a]	6.1±0.4[b]	2.8±0.2
Female NMRI mouse					
Control	8	161±37	25.6±8.5	12.5±5.6	1.9±0.6
Female Syrian hamster					
Control	7	129±25	15.5±3.3	9.6±0.6	1.0±0.1
Acetone	7	104±35	12.0±5.0	9.2±1.5	1.5±0.4[c]
PEITC	8	208±36[b,d]	29.1±4.9[b,d]	12.6±0.8[b,c]	1.9±0.5[c]

[a,b]Significantly different from control animals. (a) $P < 0.05$; (b) $P < 0.001$.
[c,d]Significantly different from acetone-treated animals. (c) $P < 0.05$; (d) $P < 0.001$.
Viability of isolated perfused segments was assessed by its ability to transport water and accumulate glucose in the absorbate. Glucose and radioactivity accumulation were calculated as the quotient $c_{absorbate}/c_{perfusate}$; water transport was calculated in µl/cm/2 h from total water absorption and segment length. Animals were treated by starvation, with acetone or PEITC as described in Materials and methods. For statistical comparison values were compared to those from untreated animals; PEITC-treated animals were also compared to the acetone-treated group.

In this study we compare intestinal NNK metabolism in rats (19) with mice and hamsters, which have been widely used for studies on NNK carcinogenesis (1,22–24). In order to avoid problems encountered with microsomal studies (for a review see 25) we used the model of isolated perfused jejunal segments as described by Richter and Strugala (26). This method retains intact intestinal walls, allows separate collection of the fluid transported across the intestinal barrier and determination of metabolite formation and distribution of the products between the serosal (absorbate) and luminal sites (perfusate). It has been successfully employed for metabolism studies of NNK (19,20) and other nitrosamines (27–29). Since in rat jejunal segments the most pronounced induction was achieved by starvation or acetone treatment, we investigated the effects of these inducers in male NMRI mice and female Syrian hamsters. The chemopreventive

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Table II. Intestinal NNK metabolism by jejunal segments from male and female Sprague-Dawley rats and NMRI mice and female Syrian hamster

Species	n	Hydroxy acid	Keto acid	Keto alcohol	NNAL N-oxide	NNK N-oxide	NNAL	Total metabolism
Female SD rat	19	0.7±1.2	1.1±2.0	2.7±2.7	1.8±2.5	7.7±7.3	12.2±13.8	26.2±17.0
		(2.7%)	(4.2%)	(10.3%)		(29.4%)	(46.6%)	
Male SD rat	8	0.2±0.2	3.4±1.3[a]	1.8±1.6	0.6±0.5	6.3±4.7	44.1±18.1[b]	56.6±15.5[b]
		(0.4%)	(6.0%)	(3.2%)	(1.1%)	(11.2%)	(78.2%)	
Female NMRI mouse	8	9.6±1.2[b,d]	39.9±2.7[b,d]	2.1±0.7	7.2±0.8[b,d]	25.7±4.3[b,d]	4.3±0.8[d]	88.5±4.0[b,d]
		(10.8%)	(44.9%)	(2.4%)	(8.1%)	(28.9%)	(4.8%)	
Male NMRI mouse	7	2.9±2.3[c,e,f]	34.8±7.4[b,d]	2.9±1.2	3.2±3.0[c,e]	20.3±1.3[a,d]	4.9±2.9[d]	68.8±10.8[b,f]
		(3.9%)	(50.6%)	(4.2%)	(4.7%)		(29.5%)	(7.1%)
Female Syrian hamster	8	10.1±1.8[b,d,h]	12.6±2.9[b,d,f,h]	5.0±1.0[a,c,f,g]	26.9±7.8[b,d,f,h]	22.3±1.3[b,d]	9.5±3.4[d,e,g]	86.4±11.0[b,d,g]
		(11.7%)	(14.6%)	(5.8%)	(31.1%)	(25.8%)	(11.0%)	

[a,b]Significantly different from female SD rats. (a) $P < 0.05$; (b) $P < 0.001$.
[c,d]Significantly different from male SD rats. (c) $P < 0.05$; (d) $P < 0.001$.
[e,f]Significantly different from female NMRI mouse. (e) $P < 0.05$; (f) $P < 0.001$.
[g,h]Significantly different from male NMRI mouse. (g) $P < 0.05$; (h) $P < 0.001$.
Metabolites in absorbates were determined by HPLC. Average values of radioactivity found in individual metabolite peaks from 7 to 19 independent perfusion experiments are given as mean ± SE. Numbers in parentheses indicate the amount of individual metabolite as percentage of total metabolites formed. Values for jejunal segments from male and female Sprague–Dawley rats (SD rats) are taken from ref. 19.

agent phenethylisothiocyanate (PEITC) reduces NNK-induced lung tumors in mice (30) and rats (31). It decreases α-hydroxylation and *N*-oxidation, but not keto reduction, of NNK in lung microsomes (32). To allow a better comparison of PEITC-related effects in mouse and hamster jejunum with the results obtained in rats (19) these inhibition experiments were conducted using intestinal segments from acetone-induced animals.

Materials and methods

Chemicals

[1-^{14}C]NNK (29 Ci/mol) was purchased from Chemsyn Science Laboratories (Lenexa, KS); it was >98% pure, but contained trace quantities of a contaminant eluting with hydroxy acid. Unlabeled NNK metabolite standards were a gift from D.Hoffmann (American Health Foundation, Valhalla, NY). PEITC was from Aldrich (Steinheim, Germany). Solvents for chromatography were HPLC grade (Merck, Darmstadt, Germany). All other chemicals were of either HPLC or analytical grade and obtained from Sigma Chemicals (Taufkirchen, Germany) or Merck (Darmstadt, Germany).

Animals

Male and female NMRI mice (20–25 g) were obtained from Interfauna (Tuttlingen, Germany). Female Syrian golden hamsters (100–150 g) were from Charles River (Sulzfeld, Germany). Animals were housed in metal cages in groups of five (hamsters) or 10 (mice) under controlled light and humidity and had free access to drinking water and pelleted food: mice, Alma H1003 Laboratory Chow (F.Borzenhardt KG, Kempten, Germany); hamsters, Trockenfutter V, Alleinfutter für MS-Haltung (Sniff, Soest, Germany). All animal experiments were approved by the Government of Upper Bavaria (AZ 211-2531-130/87 and 211-2531-53/92).

Pretreatment of animals

Acetone (8% solution in tap water) was given for 3 days. For induction by starvation (mice only) food was withdrawn for 48 h. In inhibition experiments with PEITC animals received 8% acetone in the drinking water for 3 days and additionally PEITC (25 μmol/kg/day, dissolved in sunflower oil) by oral gavage for 3 consecutive days.

Perfusion of intestinal segments

The *in vitro* perfusion technique of surviving intestinal segments has been described in detail elsewhere (26). Briefly, ~10 cm jejunal segments adjacent to the duodenum were prepared under ether anesthesia. They were perfused at 37°C from the mucosal side in an all glass perfusator with 50 ml Tyrode's solution (137 mM NaCl, 2.68 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 15 mM glucose, pH 7.4) after gassing with 95% O_2, 5% CO_2. The solution was circulated at 60 ml/min by a stream of 95% O_2, 5% CO_2. NNK (1.45 μCi/segment, 1 μmol/l final concentration) was added in 100 μl water. After 2 h perfusion the absorbed fluid (absorbate) and the remaining perfusate were weighed. The intestinal segments were rinsed with water and length and wet weight were determined. Total radioactivity was determined in aliquots from absorbate and perfusate. The accumulation of the radioactivity in the absorbate was calculated as $d.p.m._{absorbate}/d.p.m._{perfusate}$ (serosa/mucosa quotient S/M).

Viability of the intestinal segments was checked by measuring water transport and active glucose accumulation in the absorbate (Glucose GOD-Perid kit; Boehringer Mannheim, Germany). Glucose accumulation is expressed as the quotient of the glucose concentrations in absorbate and perfusate ($c_{absorbate}/c_{perfusate}$). Jejunal segments without glucose accumulation were judged non-viable, while segments with fluid transport exceeding 0.3 ml/cm/2 h were considered leaky. In both cases the individual segments were excluded from statistical evaluation.

HPLC separation and quantitation of NNK metabolism

Absorbate and perfusate samples were assayed directly for NNK and its metabolites by a modification of the method described by Carmella *et al.* (33). Briefly, 1 ml samples were deproteinized by addition of trichloroacetic acid (4% final concentration), centrifuged and 950 μl clear supernatant injected onto a C18 reversed phase column (250 mm length, 4 mm i.d, filled with LiChrospher 60 RP18 select B, 5 μ; Merck, Darmstadt, Germany). Metabolites were eluted with a gradient of 100% A for 5 min, linear to 30% B over 25 min and linear to 100% B over 1 min (A, 20 mM phosphate buffer, pH 7.4; B, acetonitrile) at a flow rate of 0.7 ml/min. Radioactivity was detected by solid phase radioactivity monitoring (Ramona, Raytest, Straubenhardt, Germany). In individual chromatograms single peaks with 200 d.p.m. (0.2% of the injected radioactivity) could be quantitated. Metabolites were identified by co-chromatography with unlabeled reference compounds detected by UV at 234 nm (UVD 160; Gynkotek, Germany).

Statistical evaluation

For each species and induction group eight to 10 jejunal segments were perfused in parallel. After exclusion of non-vital or leaky segments (no glucose accumulation in the absorbate or excess water transport) six to nine independent experiments could be evaluated. The mean ± SE was calculated for each metabolite, as well as for the metabolite groups α-hydroxides (sum of hydroxy acid, keto acid and keto alcohol) and *N*-oxides (sum of NNAL *N*-oxide and NNK *N*-oxide). Differences between groups were calculated using Student's *t*-test for unrelated groups.

Results

Intestinal viability and transepithelial NNK transport

The addition of 1 μmol/l NNK to the perfusate has shown no overt toxicity in proximal jejunal segments from rats (19). In uninduced NMRI mice and Syrian hamsters transepithelial water transport (97–161 μl/cm/2 h; Table I) was comparable to the values obtained in perfused rat intestinal segments. Starvation and acetone treatment stimulated net water absorption in male NMRI mice ($P < 0.001$). This increase was not reversed by additional PEITC treatment. In female Syrian

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hamsters water transport was not affected by acetone, but was increased 2-fold by additional PEITC treatment ($P < 0.001$).

Accumulation of glucose in the absorbate was used to determine the viability of isolated jejunal segments. In male NMRI mice glucose accumulation was comparable to the values reported for rats (S/M quotient 4.8 for male mice, 5.1 for male Sprague–Dawley rats), whereas jejunal segments from female NMRI mice and Syrian hamsters accumulated glucose to a much higher concentration in the absorbate (S/M quotient 12.5 in mice and 9.6 in hamsters). Total glucose transport was high in female mouse and hamster jejunum segments, mainly as a result of high glucose accumulation (Table I). Starvation and acetone and/or PEITC treatment moderately increased glucose uptake in male NMRI mice, whereas in female Syrian hamsters only the combined treatment with acetone and PEITC stimulated glucose transport.

Radioactivity accumulated 2-fold in the absorbate in mouse intestinal segments from both sexes, but not in hamster segments. Treatment with acetone increased the accumulation of radioactivity in mice (2.3-fold) and hamsters (1.5-fold) and was further enhanced by PEITC treatment (Table I).

NNK metabolism in untreated animals

Major differences in the intestinal metabolism of 1 μmol/l NNK were observed in jejunal segments from uninduced female Syrian hamsters, female and male NMRI mice and female and male Sprague–Dawley rats (Table II; rat data taken from 19). These differences included both the amount of NNK metabolized during the transepithelial transfer and the metabolite pattern found in the absorbate. Total metabolism ranged from 26.2% of the radioactivity in female rats to 88.5% in female mice and 86.4% in hamsters.

In hamsters and mice only 11 and 7% of the metabolites were identified as NNAL, in contrast to rats, where NNAL was the major metabolite. NNAL *N*-oxide was present in absorbates from all three species, whereas NNAL *O*-glucuronide, which has been detected in rat bile (34) and urine (7,8) of rats, mice and monkeys, could not be detected.

N-Oxidation accounted for 12 (male Sprague–Dawley rats) to 57% (female hamsters) of the metabolites formed. Whereas in hamsters NNAL *N*-oxide and NNK *N*-oxide were found in nearly equal amounts, NNK *N*-oxide was the major *N*-oxide in mouse and rat intestine.

α-Hydroxylation was the main metabolic pathway in mice, accounting for 58% of the metabolites. This was due to the formation of keto acid, which contributed 45–50% of the metabolites. Keto alcohol was present in much lower amounts (2.4 and 4.2% of the total metabolites), comparable to those found in rats. In hamsters α-hydroxylation (32.3% of the metabolites) was significantly higher than in rats (17.2 and 9.6%; Table II), with hydroxy acid, keto acid and keto alcohol being present in equal amounts.

The differences in NNK metabolism are further emphasized by the amount of metabolites excreted into the perfusate (data not shown), with much higher amounts of NNK metabolites in the perfusate from mouse (20% of the total radioactivity) and hamster (60%) as compared to rat intestine (9%). Again, in perfusates from mouse and hamster NNAL was present in only small amounts (<5% of total metabolites formed).

Induction of intestinal NNK metabolism in male NMRI mice

Intestinal NNK metabolism in Sprague–Dawley rats is stimulated by starvation and acetone treatment (19). Therefore, male NMRI mice were starved for 2 days or treated with acetone (8% in drinking water for 3 days). The results of these experiments are summarized in Table III. Total NNK metabolism increased from 69% in untreated animals to >80% in induced mice ($P < 0.05$). Both starvation and acetone treatment stimulated the formation of NNK *N*-oxide (1.6-fold) and NNAL *N*-oxide (3-fold). NNAL formation decreased from 4.9% of the total radioactivity to 1.6% in starved mice ($P < 0.05$) and was not detectable in acetone-treated animals. Changes in α-hydroxylation by these treatments were less pronounced (Table III). Starvation had no effect on α-hydroxylation, whereas acetone induction slightly stimulated the formation of keto acid and keto alcohol.

In rat intestinal segments treatment of acetone-induced animals with PEITC reduces NNK-dependent lung tumorigenesis (30,31) and microsomal NNK metabolism (12,14,18). It reduces NNK metabolism in jejunal segments from acetone-treated rats (19). To compare the PEITC effects in mouse with results in rat intestinal segments the effect of PEITC on NNK metabolism was tested in acetone-induced animals (Table III). Treatment with PEITC did not change the extent of *N*-oxidation, but slightly reduced α-hydroxylation, due to a significant reduction in keto acid formation (from 39.[illegible] to 27.5% of the total radioactivity, $P < 0.05$). NNAL, which was absent in absorbates from acetone-treated mice, was detected again after additional PEITC treatment (2.2%).

Induction of NNK metabolism in hamsters

Due to the high NNK metabolism in control hamsters treatment with acetone had only minor effects on intestinal NNK metabolism (Table IV). Overall metabolism increased from 86.4 to 94.5% (not significant). The small amount of NNAL found in control animals decreased further from 11.0 to 5.4% of the metabolites ($P < 0.05$), with a corresponding increase in NNAL *N*-oxide formation (1.4-fold, $P < 0.05$). Both the total amount of α-hydroxides and the product distribution were unaffected by acetone treatment.

After combined treatment with acetone and PEITC overall NNK metabolism was significantly increased as compared with control hamsters ($P < 0.05$). No decrease in α-hydroxylation or *N*-oxidation could be seen. It should be noted, however, that the amount of NNAL increased after PEITC treatment, but did not reach the level observed in untreated hamsters.

Discussion

In this report we describe differences in intestinal NNK metabolism between rats, hamsters and mice. In uninduced female mice and hamsters total metabolism exceeds 85%, whereas in male mice and rats ~35% and in female rats nearly 75% of the radioactivity in the absorbate is identified as NNK. Major species differences are also evident in the metabolite pattern. Acidic products, mainly keto acid, were predominant in mice and in hamsters *N*-oxidation accounted for most intestinal metabolism. NNAL, which itself is a potent carcinogen, is the main product found in rat jejunum, but is detected only in small amounts in absorbates from mouse and hamster intestinal segments. Figure 2 shows the relative contribution of the major pathways of intestinal NNK metabolism in these species.

The sex differences in NNK metabolism observed in rats were mainly restricted to NNAL formation, whereas α-hydroxylation and *N*-oxidation did not differ in male and female rats or mice. As in mouse jejunum, NNAL formation is limited in hamsters, accounting for <11% of the metabolites. In order to minimize

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Table III. Influence of starvation, acetone or PEITC treatment on the intestinal metabolism of NNK by male NMRI mice

Treatment	n	Hydroxy acid	Keto acid	Keto alcohol	NNAL N-oxide	NNK N-oxide	NNAL	Total metabolism
Control	7	2.7±2.3 (3.9%)	34.8±7.4 (50.6%)	2.9±1.2 (4.2%)	3.2±3.0 (4.7%)	20.3±6.2 (29.5%)	4.9±2.9 (7.1%)	68.8±10.8
Starved	8	4.2±2.3 (5.0%)	33.7±5.6 (40.0%)	2.0±0.8 (2.4%)	9.6±1.7[b] (11.4%)	33.2±3.7[b] (39.4%)	1.6±1.0[a] (1.9%)	84.3±9.0[a]
Acetone	8	3.4±1.0 (3.8%)	39.7±4.5[c] (44.1%)	3.2±0.6[c] (3.6%)	9.2±0.8[b] (10.2%)	34.5±7.0[b] (38.3%)	nd	90.1±11.2[a]
Acetone + PEITC	9	5.0±1.9[a] (5.9%)	27.5±7.7[d] (32.5%)	4.1±2.2[c] (4.8%)	10.8±4.6[a] (12.8%)	35.1±12.1[a] (41.4%)	2.2±2.2[d] (2.6%)	84.7±10.1[a]

[a,b]Significantly different from control mice, (a) $P < 0.05$; (b) $P < 0.001$.
[c]Significantly different from starved mice, $P < 0.05$.
[d]Significantly different from 3d acetone-treated mice, $P < 0.05$.
nd, Metabolite not detected (< 0.2% of total radioactivity).
Male NMRI mice were induced by starvation, acetone or acetone plus PEITC as described under Materials and methods. Metabolites in absorbates were determined by HPLC from 7–9 independent perfusion experiments. Numbers in parentheses indicate the amount of individual metabolite as a percentage of the total metabolites formed.

Table IV. Influence of acetone and PEITC on the intestinal metabolism of NNK by female Syrian hamsters

Treatment	n	Hydroxy acid	Keto acid	Keto alcohol	NNAL N-oxide	NNK N-oxide	NNAL	Total metabolism
Control	8	10.1±1.8 (11.7%)	12.6±2.9 (14.6%)	5.0 ± 1.0 (5.8%)	26.9 ± 7.8 (31.1%)	22.3 ± 1.3 (25.8%)	9.5 ± 3.4 (11.0%)	86.4 ± 11.0
Acetone	6	11.5±2.7 (12.2%)	13.0±2.9 (13.8%)	4.1 ± 1.4 (4.3%)	37.6 ± 1.2[a] (39.8%)	23.0 ± 2.7 (24.3%)	5.1 ± 1.0[a] (5.4%)	94.5 ± 2.5
Acetone + PEITC	8	8.7±3.8 (9.1%)	14.7±2.1 (15.3%)	6.7 ± 1.9[a,b] (7.0%)	35.5 ± 5.4[a] (36.9%)	23.2 ± 3.5 (24.1%)	7.3 ± 4.6 (7.6%)	95.9 ± 2.8[a]

[a]Significantly different from control hamsters, $P < 0.05$.
[b]Significantly different from acetone-induced hamsters, $P < 0.05$.
For explanation see legend to Table III.

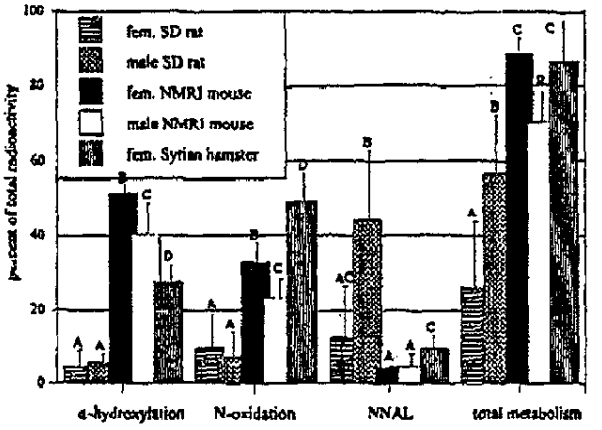

Fig. 2. Comparison of jejunal NNK metabolism in rats, mice and hamsters. NNK metabolites in absorbates from female and male Sprague–Dawley rats, female and male NMRI mice and female Syrian hamsters were determined by HPLC chromatography. α-Hydroxylation comprises the sum of hydroxy acid, keto acid and keto alcohol. N-oxidation is comprised of the sum of NNAL-N-oxide and NNK-N-oxide. All values indicate the percentage of the total radioactivity present in the samples. Different letters indicate statistically significant differences between groups. Data for male and female SD rats are taken from ref. 19.

the number of animals we did not expect additional information from male hamster jejunum segments.

Comparable differences have been observed in other organs. Mouse lung microsomes form NNAL in only minute amounts (13), whereas in rat and hamster lung microsomes NNAL accounts for 16–50% of NNK metabolism (14–16). The low NNAL formation in mouse as compared with rat or hamster lung is similar to the results obtained with jejunal segments.

Little information is available regarding the carcinogenic activity of oral NNK. In a few carcinogenicity studies in rats (35,36) and hamsters (37) 2.5–25 μmol/l NNK has been added to the drinking water. This resulted in lung, liver, nasal cavity and pancreatic tumors in rats, whereas in hamsters no tumors were observed. This correlates well with intestinal metabolism. In hamsters <20% of NNK is absorbed as NNK or NNAL, strongly reducing the carcinogenic burden. On the other hand, in rat intestinal segments >80% of radioactivity is absorbed as NNK or NNAL, inducing mainly lung tumors.

Oral application of NNK and NNAL induces pancreatic and islet cell tumors in rats (35). The frequency of pancreatic tumors after oral application of NNK was 25% of the tumors observed with NNAL. Intestinal formation of NNAL in male Fischer F344 rats as used in these bioassays (35,36) is high and approaches 50% of absorbed radioactivity (19), leading to absorption of large amounts of NNAL. Therefore, induction of pancreatic carcinomas in the rat after oral application of NNK may be explained by intestinal formation of NNAL.

In mice 300 μmol/l NNK induced lung adenomas only (21). At high substrate concentrations the percentage of NNK metabolism during absorption is lower, allowing more NNK to be absorbed. Perfusion experiments with 10 μmol/l NNK in female Sprague–Dawley rats indicated a 50% decrease in the percentage of NNK metabolized during transepithelial transfer, although the total amount of NNK metabolites formed increased ~5-fold. No differences in the metabolite pattern

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were observed (Schulze, Malone and Richter, unpublished observations). This indicates a concentration-dependent decrease in intestinal NNK metabolism, as has been observed with other nitrosamines (27).

Starvation or acetone treatment are potent inducers of cytochrome P450 IIE1 in the liver (38). This isozyme has been detected in the jejunum of induced rats (39). Both starvation and acetone treatment strongly increase NNK metabolism in intestinal segments from Sprague–Dawley rats (19). The resulting metabolite distribution indicates a similar stimulation of all three metabolic pathways. A similar, although smaller, induction, with an increase in *N*-oxidation only, was also seen in mouse and hamster jejunum, indicating a similar response of all three species to acetone induction.

The activation of NNK to potentially genotoxic intermediates by α-hydroxylation in the intestine does not necessarily lead to induction of intestinal tumors. In the intestine mucosal epithelial cells are constantly replaced. Stem cells contain no or very low amounts of cytochrome P450. Intestinal cytochrome P450 enzymes are expressed during maturation and are most prominent in non-dividing, apical enterocytes (40). Therefore, intestinal metabolism and potential genotoxic damage is restricted to mature upper epithelial cells, which do not further divide and are replaced after a few days. Tumor induction by activation of carcinogens in the intestinal wall is, therefore, unlikely, as is indicated by the low rate of intestinal tumors in both animals and man (41,42). Both α-hydroxylation and *N*-oxidation of NNK, therefore, are detoxifying reactions in the intestinal mucosa, as they reduce the amount of NNK available for systemic distribution. Formation of NNAL, on the other hand, allows absorption of a nitrosamine with a longer serum half-life (43) and which can be reoxidized to NNK *in vivo* (44), thus increasing the carcinogenic burden.

PEITC has been proposed as a chemoprotective agent. It reduces the incidence and multiplicity of NNK-induced lung tumors in mice and rats in a dose-dependent manner (30,31). Low PEITC concentrations block DNA methylation (31) and microsomal α-hydroxylation and *N*-oxidation, but not NNAL formation (14,45). Whereas addition of PEITC to microsomal incubations results in competitive enzyme inhibition, results from *in vivo* treatment of experimental animals also suggests irreversible enzyme inhibition (45).

In acetone-induced rats additional treatment with PEITC effectively inhibited intestinal α-hydroxylation and *N*-oxidation, with a concomitant increase in NNAL formation (19). In mouse and hamster intestine only small changes in NNK metabolism could be observed. While no reduction in *N*-oxide formation was seen in either mice or hamster, α-hydroxylation was reduced in mice. The small increase in NNAL formation did not reach statistical significance, but is in line with the increase observed in rats (19).

The effect of PEITC treatment in rats resulted in uptake of increased amounts of carcinogenic nitrosamines, by reducing the activity of the detoxifying pathways (19). This must be considered as a toxifying shift in NNK metabolism. The increased uptake of NNAL may be responsible for formation of pancreatic tumors in rats. In hamster and mice PEITC had only small (mice) or no (hamster) influence on the extent of NNK metabolism nor metabolite distribution. PEITC-related effects in the jejunum of mice do not explain its chemoprotective activity, which seems to be restricted to the lung (31).

In conclusion, jejunal segments from rats, mice and hamsters display differences in NNK metabolism. This includes both total metabolism and the metabolite composition. In mice and hamsters a high intestinal turnover in untreated animals limits the inductive effects of starvation and acetone. As opposed to experiments in rats, *in vivo* gavage of the chemopreventive agent PEITC has only marginal effects in mice and hamsters.

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